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Clonal Nature of *Salmonella typhi* and Its Genetic Relatedness to Other Salmonellae as Shown by Multilocus Enzyme Electrophoresis, and Proposal of *Salmonella bongori* comb. nov.

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Crude cell extracts of 26 isolates of *Salmonella* serotype typhi (*S. typhi*) and 48 other *Salmonella* isolates representing 28 serotypes and seven DNA hybridization subgroups were analyzed for electrophoretic variants of 24 metabolic enzymes by starch gel electrophoresis. All strains of *S. typhi* had identical isoenzyme patterns, indicating that they were a single clone. All of the enzymes detected in the remaining strains were polymorphic, and the degree of genetic variation was quite high. The average number of alleles per enzyme locus was 4.7, and the mean genetic diversity per locus was 0.556. Thirty-two distinct allele profiles, or electrophoretic types (ETs), were found in these 48 strains of *Salmonella* serotypes other than *S. typhi*. Analysis of the genetic relationships of the ETs to each other showed that, with one exception, the ETs formed subgroups that were consistent with the subgroupings based on DNA hybridization studies. ET profiles were not always linked to specific serologic patterns. These data show that multilocus enzyme electrophoresis has a potential application in epidemiologic and taxonomic studies of salmonellae, although it is not differential for *S. typhi*. We also propose a new species, *Salmonella bongori* comb. nov., a new combination based on the elevation of *Salmonella choleraesuis* subsp. *bongori* to the level of species.

Typhoid fever is still an important infectious disease in most of the underdeveloped world; however, the incidence of typhoid fever in the United States has declined for several decades, presumably because of good sanitation practices and treatment of carriers. Cases do occur (6), and there is a continuing need to identify isolates of epidemiologic importance. The primary methods used for differentiating strains of *Salmonella* serotype typhi (*S. typhi*) are phage typing (17), biotyping (27), and colicin production (21). Of these, phage typing has proved the most useful (16); however, 4 to 5% of isolates are nontypeable by this method, and a large number of isolates (up to 25%) are of a single phage type. All of the techniques used to differentiate strains of *S. typhi* probably measure very-short-term evolutionary distances. For example, a strain of *S. typhi* phage type F1 can change to phage type F2 through the acquisition of a single prophage, b2 (1). For this reason we wanted to study strains of the typhoid bacillus and other *Salmonella* strains with a technique that would detect greater evolutionary distance. Strains of *S. typhi* have been found to be highly related by DNA-DNA hybridization (7, 18, 19) but have not been studied by techniques, such as isoenzyme electrophoresis (29), that measure more subtle genetic differences.

The number of isolates of other *Salmonella* serotypes reported via the *Salmonella* surveillance system has increased in the last 30 years (6). This increase is thought to reflect a real change in the incidence of disease, but the reasons for the increase are not well understood. Salmonellae can be divided into subgroups by standard immunologic and biochemical methods (9), which have epidemiologic value, but the evolutionary significance of these phenotypic differences has been redefined (7, 19, 20). There is still an interest in the development of other methods for differentiating *Salmonella* isolates for taxonomic and epidemiologic studies.

Multilocus enzyme electrophoresis has been used to study the genetic diversity of a variety of bacteria (5, 13, 22–25, 28, 30, 31) and has been applied to investigations of outbreaks of Legionnaires disease (8), *Haemophilus influenzae* type b (4), and *Escherichia coli* strains that cause a *Shigella*-like disease (30). We report here the use of multilocus enzyme analysis for the study of *S. typhi* and representative strains of the seven *Salmonella* subgroups defined by DNA-DNA hybridization (7, 18–20).

MATERIALS AND METHODS

Nomenclature. Although all *Salmonella* strains have been found to be highly related by DNA-DNA hybridization, we prefer to artificially treat the named serotypes as if they are species. In this system *Salmonella* serotype typhi is written as *S. typhi*. This is in keeping with the nomenclature most frequently used in the literature.

Bacterial strains. The *Salmonella* strains used in this study (Table 1) were obtained from the World Health Organization National Salmonella Center, housed in the Enteric Bacteriology Section, Enteric Diseases Branch, Center for Infectious Diseases, Centers for Disease Control. All *S. typhi* strains are clinical strains, except for strain Ty-21a, which is a proposed vaccine strain (12). All strains were serotyped, and all *S. typhi* strains were phage typed by methods previously described (9, 11). The DNA-DNA hybridization subgroups of the strains are those described by Crosa et al. (7) and Le Minor et al. (18–20).

Enzyme extract preparation. Strains were grown overnight at 35°C on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.). Cells from fi e plates (20 by 150 mm) of

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TABLE 1. Characteristics of *Salmonella* serotypes used in this study

Serotype	CDC strain no.[a]	DNA subgroup	O group	Antigenic formula	Phage type	Origin	ET
S. typhi	9000-85	I	D	9,12,Vi:d:−	40	Egypt	1
S. typhi	9001-85	I	D	9,12,Vi:d:−	N	Egypt	1
S. typhi	9002-85	I	D	9,12,Vi:d:−	D1-N	Egypt	1
S. typhi	9003-86	I	D	9,12,Vi:d:−	53	Egypt	1
S. typhi	9004-86	I	D	9,12,Vi:d:−	T	Egypt	1
S. typhi	9005-86	I	D	9,12,Vi:d:−	E2	Egypt	1
S. typhi	9006-86	I	D	9,12,Vi:d:−	Des-ViJ	Egypt	1
S. typhi	9007-86	I	D	9,12,Vi:d:−	F1	Egypt	1
S. typhi	9008-86	I	D	9,12,Vi:d:−	D6	Egypt	1
S. typhi	9009-86	I	D	9,12:d:−	Nontypeable	Egypt	1
S. typhi	107-82	I	D	9,12,Vi:d:−	37	Columbia	1
S. typhi	382-82	I	D	9,12,Vi:d:−	M1	Marshall Island	1
S. typhi	1794-82	I	D	9,12,Vi:d:−	O	United States	1
S. typhi	9032-85	I	D	9,12,Vi:d:−	D1	Taiwan	1
S. typhi	1707-81	I	D	9,12,Vi:d:−	C1	Liberia	1
S. typhi	674-80	I	D	9,12,Vi:d:−	A	Mauritius	1
S. typhi	3273-76	I	D	9,12,Vi:d:−	46	American Samoa	1
S. typhi	1196-74	I	D	9,12,Vi:d:−	Mex S	Mexico	1
S. typhi	3434-73	I	D	9,12,Vi:d:−	G1	Peru	1
S. typhi	3304-78	I	D	9,12,Vi:d:−	J1	Italy	1
S. typhi	3137-78	I	D	9,12,Vi:d:−	K1	India	1
S. typhi	1994-78	I	D	9,12,Vi:d:−	27	Lebanon	1
S. typhi	934-79	I	D	9,12,Vi:d:−	E1	Australia	1
S. typhi	9228-77	I	D	9,12,Vi:d:−	35/38	El Salvador	1
S. typhi	3815-73	I	D	9,12,Vi:d:−	28	Unknown	1
S. typhi	Ty 21a[b]	I	D	9,12:d:−	W	Unknown	1
S. typhimurium	2360-79	I	B	1,4,5,12:i:1,2		North Dakota	2
S. typhimurium	1649-78	I	B	1,4,5,12:i:1,2		Wyoming	3
S. typhimurium	3280-78	I	B	1,4,5,12:i:1,2		Nebraska	4
S. typhimurium	1689-77	I	B	1,4,5,12:i:1,2		Missouri	3
S. typhimurium	391-76	I	B	1,4,5,12:i:1,2		Arkansas	3
S. typhimurium	179-87	I	B	1,4,5,12:i:1,2		Utah	3
S. typhimurium	331-86	I	B	1,4,5,12:i:1,2		Colorado	3
S. typhimurium	137-85	I	B	1,4,5,12:i:1,2		New York	5
S. typhimurium	655-84	I	B	1,4,5,12:i:1,2		New Hampshire	3
S. typhimurium	1119-83	I	B	1,4,5,12:i:1,2		Maryland	3
S. heidelberg	4689-70	I	B	1,4,5,12:r:1,2		Georgia	6
S. heidelberg	302-56	I	B	1,4,12:r:1,2		Louisiana	7
S. heidelberg	5741-72	I	B	1,4,12:r:1,2		Arkansas	7
S. heidelberg	1384-67	I	B	1,4,12:r:1,2		Virginia	7
S. heidelberg	1827-63	I	B	1,4,5,12:r:1,2		Ohio	7
S. heidelberg	3787-53	I	B	1,4,5,12:r:1,2		Washington	7
S. hadar	482-82	I	C2	6,8:z_{10}:e,n,x		South Dakota	8
S. hadar	2998-81	I	C2	6,8:z_{10}:e,n,x		Minnesota	8
S. hadar	585-86	I	C2	6,8:z_{10}:e,n,x		Tennessee	8
S. hadar	68-87	I	C2	6,8:z_{10}:e,n,x		Massachusetts	8
S. agona	1793-74	I	B	1,4,12:f,g,s:−		Peru	9
S. agona	3531-75	I	B	1,4,12:f,g,s:−		New Hampshire	9
S. agona	1290-77	I	B	1,4,12:f,g,s:−		North Dakota	9
S. agona	1580-79	I	B	1,4,12:f,g,s:−		Oregon	9
S. paratyphi A	1072-82	I	A	1,2,12:a:−		South Carolina	10
S. choleraesuis	290-71	I	C1	6,7:c:1,5		California	11
S. typhisuis	STK-38[b]	I	C1	6,7:c:1,5		Unknown	12
S. gallinarum	2950-79	I	D	1,9,12:−:−		Ecuador	13
S. pullorum	2385-70	I	D	9,12:−:−		North Dakota	14
S. sendai	STK-71[b]	I	D	1,9,12:a:1,5		Unknown	15
S. phoenix	696-84	II	X	47:b:1,5		Iowa	16
58:d:z_6	151-85	II	58	58:d:z_6		Massachusetts	17
S. setubal	42-87	II	60	60:g,m,t:z_6		Mississippi	18
18:z_4,z_{32}:−[c]	98-84	IIIa	K	18:z_4,z_{32}:−		Connecticut	19
48:z_4,z_{24}:−[c]	187-87	IIIa	Y	48:z_4,z_{24}:−		Tennessee	20
62:z_{36}:−[c]	409-85	IIIa	62	62:z_{36}:−		California	21
38:(k):z_{35}	678-84	IIIb	P	38:(k):z_{35}		California	22
50:k:z[c]	156-87	IIIb	Z	50:k:z		Oregon	23
61:k:1,5,(7)[c]	2565-81	IIIb	61	61:k:1,5,(7)		Georgia	24
43:z_{29}:−	64-85	IV	U	43:z_{29}:−		Missouri	25
44:z_{36}:−	525-86	IV	V	44:z_{36}:−		Georgia	26

Continued on following page

TABLE 1—*Continued*

Serotype	CDC strain no.[a]	DNA subgroup	O group	Antigenic formula	Phage type	Origin	ET
S. wassenaar	121-87	IV	Z	50:g,z_{51}:−		Massachusetts	27
S. malawi	327-80	V	66	66:z_{65}:−		France	28
S. maregrosso	235-77	V	66	66:z_{35}:−		France	29
S. brookfield	750-72	V	66	66:z_{41}:−		Illinois	30
11:b:1,7	4603-68	VI	F	11:b:1,7		France	31
S. ferlac	1411-60	VI	H	1,6,14,25:a:e,n,x		England	32
S. vrindaban	2083-62	VI	W	45:a:e,n,x		England	33

[a] The last two digits are the year of isolation unless stated otherwise.
[b] Year of isolation unknown.
[c] Formerly called *Arizona hinshawii* or *Salmonella arizonae*.

each strain were harvested into 10 mM potassium phosphate-buffered saline (pH 7.2), concentrated by centrifugation (12,000 × *g* for 15 min), and suspended in 1.5 ml of cold 10 mM Tris hydrochloride (pH 6.8) containing 1 mM EDTA and 0.5 mM NADP. The suspensions were kept in an ice bath during most of the following procedure. To each suspension was added 1 ml of glass beads (75 to 150 μm; G-3753; Sigma Chemical Co., St. Louis, Mo.), and the suspensions were vortexed at high speed on a heavy-duty mixer (S82202; American Scientific Products, McGaw Park, Ill.) for three 1-min periods separated by 1-min intervals in the ice bath. Cell debris was removed by centrifugation (25,000 × *g* for 30 min), and the supernatants were filtered (0.22-μm-pore Millex-GV filters; Millipore Corp., Bedford, Mass.) and stored as aliquots at −80°C for later analysis. Preliminary studies with salmonellae indicated that this procedure yielded the maximum soluble protein (4 to 5 mg/ml) with cells of different strains.

Electrophoresis and enzyme analysis. Individual cell extracts were adsorbed onto filter paper wicks (5 by 17 mm) which were inserted vertically into an 11.5% starch gel (Starch-Hydrolyzed; Connaught Laboratories Ltd., Willowdale, Ontario, Canada) containing 0.023 mM Tris–0.0052 mM citric acid (pH 8.0). The buffer for both electrodes was 0.687 mM Tris–0.157 mM citric acid (pH 8.0), and electrophoresis was conducted with cooling (2.8°C) at 135 V for the length of time needed to move a marker dye (0.05% bromophenol blue in 80% glycerol–50 mM Tris hydrochloride [pH 8.0]) 10 cm toward the anode. This required about 6 h for most gels. Gel slices (2 mm thick) were stained for the enzymes listed in Table 2 by the methods described by Selander et al. (29). NADPH diaphorase (EC 1.6.2.2) and adenosine deaminase (EC 3.5.4.4) were stained by the methods of Harris and Hopkinson (15). Electrophoretic variants of each enzyme were considered alleles of that enzyme and were assigned different ascending allele numbers based on their increasing mobility toward the anode. Each strain was characterized by a profile of alleles for the enzymes of that strain, and each unique allele profile was designated an electrophoretic type (ET) and given an assigned number.

Statistical methods. Genetic diversity at each enzyme locus (the degree to which each enzyme locus varied) was calculated as described by Selander et al. (29). Mean genetic diversity per locus was calculated as the arithmetic average

TABLE 2. Characteristics of 24 *Salmonella* enzyme loci

EC no.	Symbol	Enzyme	No. of alleles[a]	Genetic diversity[b]
1.1.1.17	M1P	Mannitol-1-phosphate dehydrogenase	4	0.544
1.1.1.37	MDH	Malate dehydrogenase	4	0.551
1.1.1.40	ME	"Malic" enzyme	3	0.174
1.1.1.42	IDH	Isocitrate dehydrogenase ($NADP^+$)	4	0.411
1.1.1.44	6PG	Phosphogluconate dehydrogenase	5	0.491
1.1.1.49	G6P	Glucose-6-phosphate dehydrogenase	5	0.674
1.4.1.4	GDH	Glutamate dehydrogenase ($NADP^+$)	4	0.366
1.6.99.1	DA1	Diaphorase (NADPH) 1	7	0.686
1.6.99.1	DA2	Diaphorase (NADPH) 2	5	0.633
1.15.1.1	IP1	Indophenol oxidase 1 (superoxide dismutase)	4	0.593
1.15.1.1	IP2	Indophenol oxidase 2 (superoxide dismutase)	6	0.581
2.4.2.1	NSP	Purine-nucleoside phosphorylase	6	0.693
2.6.1.1	GOT	Glutamic-oxaloacetic transaminase	4	0.592
2.7.4.3	ADK	Adenylate kinase	4	0.600
2.7.5.1	PM1	Phosphoglucomutase 1	3	0.492
2.7.5.1	PM2	Phosphoglucomutase 2	3	0.316
3.1.3.2	AP1	Acid phosphatase 1	3	0.534
3.1.3.2	AP2	Acid phosphatase 2	3	0.568
3.4.11.11	PEP	Peptidase (Phe-Leu)	5	0.763
3.5.4.4	ADA	Adenosine deaminase	5	0.625
4.2.1.2	FUM	Fumarase	8	0.818
4.2.1.3	ACO	Aconitase	6	0.504
5.3.1.8	MPI	Mannose-6-phosphate isomerase	5	0.599
5.3.1.9	PGI	Phosphoglucoseisomerase	6	0.540

[a] Mean, 4.7.
[b] Mean, 0.556.

TABLE 3. Number of alleles for 33 ETs of salmonellae

ET	DNA subgroup	No. of isolates	Serotype	No. of alleles at the following 24 enzyme loci[a]:																		
				M1P	MDH	ME	IDH	6PG	G6P	GDH	DA1	DA2	IP1	IP2	NSP	GOT	ADK	PM1	PM2	AP1	AP2	PEP	ADA
1	I	26	*S. typhi*	5	5	4	4	5	5	5	6	6	5	4	5	6	5	0	4	4	4	5	5
2	I	1	*S. typhimurium*	5	5	4	4	6	7	6	6	6	3	5	3	6	5	6	4	0	4	5	7
3	I	7	*S. typhimurium*	5	5	4	4	6	7	6	6	6	3	5	3	6	5	6	4	0	4	7	7
4	I	1	*S. typhimurium*	5	5	4	4	5	7	6	6	6	3	5	3	6	5	6	4	0	4	7	7
5	I	1	*S. typhimurium*	7	5	4	3	5	6	6	6	6	5	5	5	6	5	6	4	0	4	5	5
6	I	1	*S. heidelberg*	5	5	4	4	6	7	6	6	6	5	3	3	6	5	6	4	0	4	7	5
7	I	5	*S. heidelberg*	5	5	4	4	6	7	6	6	6	5	3	3	6	5	6	4	0	4	7	5
8	I	4	*S. hadar*	5	5	4	4	6	7	6	6	5	5	3	5	6	5	6	4	2	4	7	6
9	I	4	*S. agona*	5	5	4	4	5	7	6	6	6	5	6	5	6	5	0	4	0	4	5	5
10	I	1	*S. paratyphi* A	7	5	5	4	5	7	6	6	6	0	0	5	6	6	0	4	2	0	8	5
11	I	1	*S. choleraesuis*	5	5	4	4	5	7	6	6	6	4	7	6	6	5	6	4	0	4	8	4
12	I	1	*S. typhisuis*	0	5	4	3	5	7	6	6	6	5	7	6	6	5	6	4	0	4	8	4
13	I	1	*S. gallinarum*	5	3	4	4	5	7	6	6	6	5	6	7	6	5	6	4	0	4	7	6
14	I	1	*S. pullorum*	5	5	4	4	5	7	6	6	6	0	0	6	0	5	0	9	0	4	7	6
15	I	1	*S. sendai*	7	5	4	4	5	7	6	6	6	5	6	5	6	5	0	4	4	0	6	0
16	II	1	*S. phoenix*	5	3	4	4	4	3	6	5	5	3	3	3	6	7	0	4	0	0	9	5
17	II	1	58:d:z_6	5	3	4	4	4	3	6	5	5	3	3	3	6	7	0	4	0	0	8	5
18	II	1	*S. setubal*	7	3	4	4	5	6	6	5	5	3	3	3	6	7	0	4	0	0	8	5
19	IIIa	1	18:z_4,z_{32}:-	7	5	4	5	5	6	0	4	6	3	3	5	5	8	0	4	0	0	8	4
20	IIIa	1	48:z_4,z_{24}:-	7	5	4	4	5	6	0	4	6	5	3	5	5	8	0	4	0	0	8	4
21	IIIa	1	62:z_{36}:-	7	5	4	4	5	6	0	4	6	3	3	4	5	8	0	4	4	0	8	4
22	IIIb	1	38:(k):z_{35}	5	3	4	4	6	4	6	6	3	3	3	3	5	5	0	4	4	0	7	5
23	IIIb	1	50:k:z	5	2	4	4	5	7	6	6	3	3	3	3	5	7	0	4	4	0	8	5
24	IIIb	1	61:k:1,5,(7)	5	3	4	4	5	7	6	6	3	3	3	3	5	7	0	4	4	0	8	5
25	IV	1	43:z_{29}:-	5	3	4	5	5	6	6	3	4	3	3	3	6	7	0	4	0	0	8	5
26	IV	1	44:z_{36}:-	3	3	4	4	5	6	6	4	5	3	3	5	6	7	0	4	0	0	8	5
27	IV	1	*S. wassenaar*	5	3	4	5	2	7	6	3	5	3	3	6	6	7	0	4	0	0	8	5
28	V	1	*S. malawi*	5	3	4	4	5	5	6	0	5	3	3	5	4	5	5	0	0	4	7	4
29	V	1	*S. maregrosso*	7	3	3	4	5	5	6	8	5	3	3	3	4	5	5	0	0	4	7	4
30	V	1	*S. brookfield*	5	3	3	4	5	5	6	8	5	3	3	3	4	5	5	0	0	4	8	5
31	VI	1	11:b:1,7	3	5	4	5	5	6	7	7	5	5	3	5	4	7	0	4	4	0	5	5
32	VI	1	*S. ferlac*	3	8	4	5	3	6	6	7	5	5	3	5	4	7	0	4	4	0	8	5
33	VI	1	*S. vrindaban*	5	5	4	4	5	6	6	0	0	5	3	5	4	7	0	4	4	0	8	5

[a] See Table 2 for explanation of enzyme abbreviations.

of the values for all of the loci. Genetic distances between pairs of strains were calculated as the proportion of weighted mismatches of alleles (29, 31), and a dendrogram showing the clustering or relatedness of the ETs was generated by the unweighted pair group method for arithmetic averages (32), using the commercial program CLUSTAN 1C (33). Genetic distances were also examined by principal coordinate analysis (14) to provide a more visual representation of the spacial relationships among the ETs.

RESULTS

Enzyme locus variation. Of the 20 enzymes that were found in the salmonellae, 4 (diaphorase, indophenol oxidase, phosphoglucomutase, and acid phosphatase) each produced two bands in the gels with most of the strains. Each band was considered to be a separate enzyme locus, for a total of 24 enzyme loci (Table 2). All of these enzymes were polymorphic. Genetic diversity among the enzyme loci was quite high, with a mean per locus of 0.556. The mean number of alleles per locus was 4.7, with a range of 3 to 8 (Table 2). A comparison of the allele profiles of the strains showed that 33 distinct allele combinations, or ETs, were present in this collection of 74 strains (Table 3).

Clonal nature of *S. typhi*. A surprising finding was that all *S. typhi* strains had identical enzyme patterns (Tables 1 and 3), suggesting that all isolates of *S. typhi*, regardless of phage type, geographic origin, or time of isolation (1973 to 1985), were members of a single clone. The other serotypes had more variation. Four ETs were found among the 10 strains of *S. typhimurium*. The *S. typhimurium* strain (137-85, Table 1) that differed the most from the other serotype typhimurium strains (ET-5, Fig. 1) was dulcitol negative, whereas the others were dulcitol positive. Two ETs were found among the six strains of *S. heidelberg* (Table 1); the four strains of *S. hadar* and the four strains of *S. agona* contained one ET each. The remaining serotypes, which were each represented by one strain, all contained unique ETs. The DNA subgroups (7, 18–20) of these strains contained multiple ETs (Tables 1 and 3), indicating divergence within these DNA subgroups.

Relationships among ETs. The genetic relationships of the ETs to each other are shown in the dendrogram in Fig. 1. At a genetic distance of about 0.66, all of the salmonellae were grouped as a single cluster. At a genetic distance of about 0.57, the *Salmonella* strains formed two clusters, one of which contained DNA subgroup V and the other of which contained all of the remaining subgroups. These data fit the reported observation that subgroup V shows the least relatedness to the other *Salmonella* subgroups by DNA hybridization (18). When the data were examined at smaller genetic distances between the ETs, the major DNA subgroups separated from one another (Fig. 1). At a distance of about 0.45, DNA subgroups I, IIIa, V, and VI were clearly separate, whereas subgroups II, IIIb, and IV were still clustered. At a genetic distance of 0.36, subgroup IIIb separated as a single cluster, but subgroups II and IV still remained linked, mainly because of the close association of ET-26 (serotype 44:z_{36}:−) with ET-18 (*S. setubal*) (Table 1). Also at this genetic distance, subgroup I was divided into three distinct clusters. The first contained only ET-1 (*S. typhi*); the second contained ET-2 through ET-9 and ET-11 through ET-14, representing a diverse group of serotypes; and the third contained ET-10 (*S. paratyphi* A) and ET-15 (*S. sendai*). DNA subgroups IIIa and IIIb (formerly the genus *Arizona*; 10) were quite distinct from each other, showing no relatedness until a genetic distance of about 0.53 (Fig. 1). DNA hybridization studies (7) have also shown that these two groups are distinct, although both groups are salmonellae.

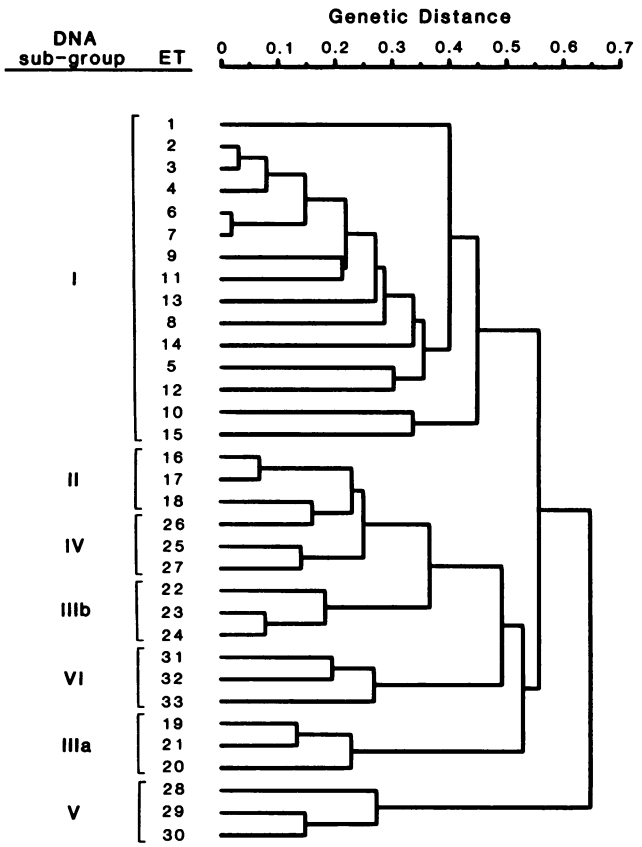

FIG. 1. Genetic relationships among 33 ETs of seven *Salmonella* DNA subgroups. The dendrogram was produced by the average-linkage method of clustering from a matrix of coefficients of weighted distance, based on 24 enzyme loci (29, 31). The ET numbers and the DNA subgroup designations are those shown in Tables 1 and 3.

The spatial relationships of the ETs are also illustrated by principal coordinate analysis of the data in three dimensions (Fig. 2). The 15 ETs that made up DNA subgroup I showed some diversity in their genetic relatedness but still formed a large separate cluster from the other groups. *S. typhi* (ET-1) is indicated by the arrow in Fig. 2. The ETs of DNA subgroups II, IIIb, and IV formed a separate cluster in the middle of the figure, reflecting their clustering in the dendrogram analysis (Fig. 1). The ETs of subgroups IIIa and VI appeared to form a very loose third cluster, while the ETs of subgroup V were grouped into a very distant fourth cluster (Fig. 2). Viewed in this way, the data show that there is an underlying four-part structure to the genetic relationships among the ETs in this collection of strains.

Relationship between ET and serotype. The ET designations of the strains were not absolutely linked to any particular serologic characteristics. The 26 *S. typhi* strains all contained a single ET, and all had identical antigenic compositions, except for the loss of the Vi antigen in two strains (Table 1). The 10 strains of *S. typhimurium*, however, contained four ETs, and the six strains of *S. heidelberg* contained two ETs (Table 1). Two of the five *S. heidelberg* strains that were ET-7 had a slightly different antigenic

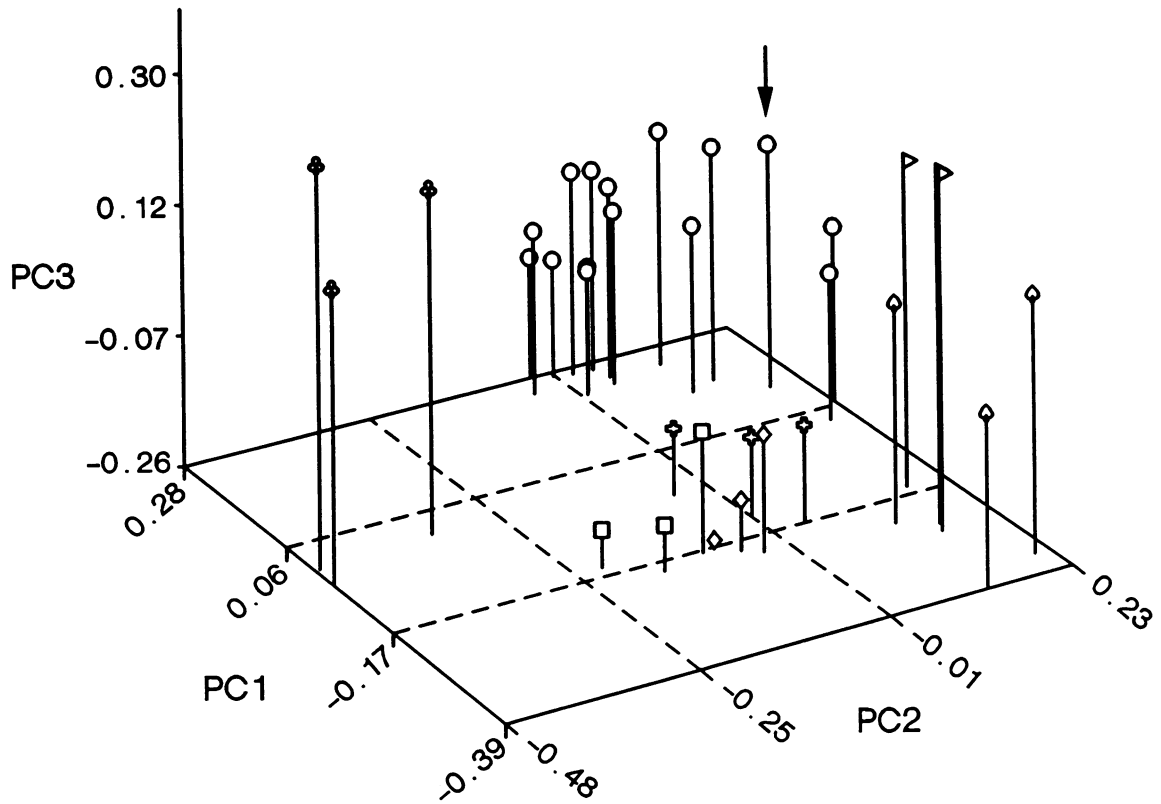

FIG. 2. Principal coordinate analysis of the ETs of *Salmonella* DNA subgroups I (circles), II (squares), IIIa (triangles), IIIb (crosses), IV (diamonds), V (clubs), and VI (spades). *S. typhi* (ET-1) is indicated by the arrow.

composition than the other three (Table 1). *S. choleraesuis* and *S. typhisuis*, which share identical serologic characteristics, contained distinctly different ETs (Tables 1 and 3).

DISCUSSION

The purpose of this study was to see if multilocus enzyme electrophoresis could differentiate strains of *S. typhi*. The data presented here show that this serotype is actually a single clone (Table 1). Previous studies have shown that strains of this serotype have identical serologic and biochemical characteristics (10, 16, 27), and the present data support these observations. The term clone is being used here in the sense suggested by Ørskov and Ørskov (26): that strains that are similar in a phenotypic or genetic sense have a common origin. The results of our study show that the multilocus enzyme method cannot be used to differentiate strains of *S. typhi* from each other; however, the present system of phage typing is still highly effective in epidemiologic studies of this organism (16, 17). We have shown, however, that multilocus enzyme analysis could have an application in epidemiologic and taxonomic studies of at least some *Salmonella* serotypes. These surprising results for *S. typhi* led us to expand our original study to include other *Salmonella* strains, to examine the genus for divergence, and to put the *S. typhi* clone into perspective within the genus.

The results show that *Salmonella* strains are genetically a highly variable group of organisms. A minimum of 33 ETs (genotypes) were found in 74 strains representing 28 serotypes, with an average of 4.7 alleles per enzyme locus and a mean genetic diversity per locus of 0.556. As expected, there was much more variation among serotypes than within serotypes. These data indicate that genetic variation in salmonellae is comparable to that found in *Haemophilus* spp. (28) and *Escherichia coli* (25), which showed genetic diversity values of 0.573 and 0.52, respectively. This degree of variation is much greater than that found in *Shigella* spp. (25), *Legionella pneumophila* (31), and *Bordetella* spp. (23), which showed genetic diversity values of 0.29, 0.313, and 0.284, respectively.

Despite the small number of strains used in this study, analysis of the relationships of these ETs to each other shows that, with one exception, multilocus enzyme electrophoresis data support the taxonomic subgroupings that have been proposed on the basis of DNA-DNA hybridization studies (7, 18–20). In our study, subgroups II and IV could not be separated, but this was mainly because strain 525-86 (ET-26) grouped with the ETs of subgroup II. No DNA-DNA hybridization data are available for this strain, but it was placed in DNA subgroup IV on the basis of its biochemical and serologic characteristics. According to our data this strain belongs in subgroup II. DNA subgroup V was the most distant from the other salmonellae, as originally reported by Le Minor et al. (18, 19). This group shares enough serologic and biochemical characteristics and DNA relatedness with salmonellae to be grouped in the genus *Salmonella* (10, 18–20), but the results of our study and DNA-DNA hybridization studies (18, 19) suggest that this group evolved significantly from all the other *Salmonella* subgroups, which are much more highly related to each other. Le Minor et al. (18, 19) reported that subgroup V shares about 55 to 60% relatedness with the other *Salmonella* subgroups, while the

other subgroups share 63 to 74% relatedness with each other.

In our study, the remaining six DNA subgroups form a large, loose cluster that appears to contain at least three distinct subclusters. Subgroups IIIa and VI appear as distinct groups when the data are displayed as a dendrogram, but by principal coordinate analysis they are clustered more closely to each other than to the other salmonellae. This is partly because the principal coordinate analysis method, by its innate nature (14), displays only 63% of the data shown in the dendrogram. When viewed in this manner, the data clearly show that these two subgroups are still distinct, but this method of analysis also shows the relationships of these subgroups to the other subgroups in a way that cannot be seen in the dendrogram. Subgroups II, IIIb, and IV form a second cluster, and the ETs of subgroup I make up the third cluster.

Subgroup I appears to be a more heterogeneous collection of serotypes that only group together at a genetic distance of about 0.45. This result was not unexpected, since this group is widely dispersed in nature and has had many chances to evolve through host adaptation and other evolutionary pressures. DNA-DNA hybridization studies of strains in this subgroup have shown that they share 79 to 103% relatedness with each other (7). This degree of shared DNA clearly groups these strains at the species level, but it also indicates that some heterogeneity exists in this subgroup.

Selander et al. (31), in a multilocus enzyme study of *L. pneumophila*, proposed that a genetic distance between 0.5 and 0.6 might serve as a cutoff point in separating one species from another. If that criterion were applied to the data in this study, DNA subgroup V would clearly be identified as a separate *Salmonella* species. Brenner (2) and Brenner et al. (3) have shown that a DNA relatedness of 55%, at less than optimal conditions, can define the members of a single species; however, the DNA relatedness of subgroup V to the other *Salmonella* subgroups is about 43%, at less than optimal conditions (18). Our results then, agree with those of Le Minor et al. (18, 19) that *Salmonella* subgroup V has evolved considerably from the other *Salmonella* subgroups. We believe that this divergence is sufficient to consider it as a separate species rather than as a subspecies. We propose that *Salmonella choleraesuis* subsp. *bongori* be elevated to the level of species in the new combination *Salmonella bongori* comb. nov. The type strain of *S. bongori* remains CIP 8233, and the description for *S. bongori* is the same as the original description given for *S. choleraesuis* subsp. *bongori* (20).

Application of multilocus enzyme electrophoresis to classify strains to the subspecies level has not been done; however, the method has demonstrated in this study and for a variety of other bacteria that a complex genetic structure can exist below the species level (5, 13, 22–25, 28, 30, 31).

ADDENDUM IN PROOF

Recently, Beltran et al. (P. Beltran, J. M. Musser, R. Helmuth, J. J. Farmer III, W. M. Frerichs, I. K. Wachsmuth, K. Ferris, A. C. McWhorter, J. G. Wells, A. Cravioto, and R. K. Selander, Proc. Natl. Acad. Sci. USA **85**:7753–7757, 1988) reported on the analysis of salmonellae by multilocus enzyme electrophoresis. They found 71 ETs in 1,527 strains representing eight serotypes of DNA subgroup I. Analysis of the genetic relatedness of these ETs demonstrated a clonal population structure similar to what we have found with strains of this DNA subgroup. They did not include strains of *S. typhi* in their study.

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